A Novel Toll-Like Receptor 9 Agonist Cooperates with Trastuzumab in Trastuzumab-Resistant Breast Tumors through Multiple Mechanisms of Action

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Abstract Purpose: Resistance to anti-HER2 monoclonal antibody trastuzumab is a relevant issue in breast cancer patients. Among the mechanisms implicated in trastuzumab resistance, increasing evidence supports a role of tumor microenvironment. We previously found that a novel toll-like receptor 9 agonist, referred to as immune modulatory oligonucleotide (IMO) and currently under clinical investigation, acts through epidermal growth factor receptor (EGFR) and shows direct antiangiogenic effects by cooperating with anti-EGFR or anti-VEGF drugs, thus interfering with cancer cells and microenvironment.

Experimental Design: In this study, we used KPL-4 and JIMT-1 trastuzumab-resistant breast cancer cells to evaluate the combination IMO plus trastuzumab as a therapeutic option for trastuzumab-resistant breast cancers.

Results: IMO inhibits KPL-4 and JIMT-1 xenografts growth and potentiates trastuzumab antitumor effect, with complete suppression of tumor growth, potent enhancement of trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity, and strong inhibition of EGFR/HER2-related signaling. In KPL-4 xenografts, IMO alone interferes with HER signal transduction, whereas trastuzumab is ineffective. IMO induces an HER-dependent signal inhibition also in vitro by modulating a functional interaction between toll-like receptor 9 and HER receptors occurring at membrane level. Finally, IMO plus trastuzumab produces a cooperative antiangiogenic effect related to suppression of endothelial HER-related signaling.

Conclusions: We showed a cooperative effect of IMO plus trastuzumab in trastuzumab-resistant breast cancers due to IMO direct antitumor and antiangiogenic activity and antibody-dependent cell-mediated cytotoxicity enhancement. Moreover, we provided first evidence of a toll-like receptor 9/HER interaction at membrane level as novel mechanism of action. Altogether, we propose IMO plus trastuzumab as an effective strategy in trastuzumab-resistant breast cancers. (Clin Cancer Res 2009;15(22):6921–30)
In the KPL-4 cell line, isolated from the malignant pleural effusion, PTEN gene copy number ratio is unaltered (6). However, in JIMT-1 cells, it has been shown that PTEN activity is unlikely to respond to trastuzumab-based therapy (9, 10).

PTEN phosphatase, the most important negative regulator of both mutations of phosphoinositide 3-kinase and loss of the phosphoinositide 3-kinase/Akt activity in trastuzumab resistance. In fact, factor-I receptor able to overactivate HER2 in resistant cells (8). Formation of heterodimers between HER2 and insulin-like growth factor-I receptor can promote resistance to trastuzumab has been also associated with increased signaling of HER2-targeted mAbs bind to circulating extracellular domain, competing away binding to membrane-bound HER2 (14). The p95HER2 protein may be generated by different, nonmutually exclusive mechanisms, including not only the extracellular domain cleavage but also alternative RNA processing (15) or alternative initiation of translation from different methionines within the HER2 sequence (16).

In the JIMT-1 cell line, established from several preclinical studies showing antitumor activity in a wide variety of tumor models, in monotherapy or in combination with traditional anticancer therapies (19–24). Based on extensive structure-activity relationship studies, synthetic toll-like receptor 9 agonists containing novel DNA structures and synthetic dinucleotide motifs, referred to as immune modulatory oligonucleotides (IMOs), have been described. IMOs have been shown to induce distinct cytokine profiles in vitro and in vivo (25, 26) and showed higher metabolic stability (27) compared with conventional toll-like receptor 9 agonists.

In preclinical tumor models, IMOs enhanced antitumor activity in combination with peptide and DNA vaccines inducing Th1-type antigen-specific antibody production and cytotoxic T-cell responses (28, 29). Previous studies with IMOs have shown potent antitumor activity in combination with chemotherapeutic agents, mAbs, and radiation (28, 30). Currently, a synthetic agonist of toll-like receptor 9, IMO-2055, is under clinical evaluation, in combination with chemotherapy and other agents in cancer patients. Although clinical development of toll-like receptor 9 agonists is very encouraging, the mechanisms by which they affect signaling proteins involved in tumor growth and angiogenesis, thus leading to tumor growth inhibition, are yet to be elucidated. Recently, we showed that an IMO acts by impairing epidermal growth factor receptor (EGFR) signaling, cooperates with anti-EGFR drugs, and boosts the antibody-dependent cell-mediated cytotoxicity (ADCC) of anti-EGFR antibodies (30). In another study, we showed that IMO interferes with tumor growth and angiogenesis also by EGFR- and ADCC-independent mechanisms, affecting endothelial cell functions (31). These new findings have opened the path to clinical studies combining toll-like receptor 9 agonists with EGFR and VEGF inhibitors in cancer patients.

Therefore, IMO shows antitumor effects through toll-like receptor 9-mediated innate, adaptive, and cell-mediated immune responses of trastuzumab in clinical practice, a number of patients will either not respond or eventually progress despite trastuzumab treatment. The rate of primary resistance to single-agent trastuzumab for HER2-overexpressing metastatic breast cancer is 66% to 88%; therefore, significant efforts have been applied to elucidate the molecular basis of resistance to trastuzumab and find therapeutic strategies potentially effective also in trastuzumab-resistant HER2-positive breast cancer (1, 3, 4). Resistance to trastuzumab is often associated with increased expression of the membrane-associated glycoprotein MUC4 that binds and stericly hinders HER2 from binding to trastuzumab, also activating the receptor through its epidermal growth factor (EGF)–like domain (5). In the JIMT-1 cell line, established from a breast cancer patient showing 

\[ \text{her2} \] gene amplification and primary resistance to trastuzumab, elevated MUC4 expression masks the trastuzumab binding epitopes of HER2 (6). Trastuzumab resistance has been also associated with increased signaling from the insulin-like growth factor-I receptor (7) or with the formation of heterodimers between HER2 and insulin-like growth factor-I receptor able to overactivate HER2 in resistant cells (8). Several evidence support the role of constitutive phosphoinositide 3-kinase/Akt activity in trastuzumab resistance. In fact, both mutations of phosphoinositide 3-kinase and loss of the PTEN phosphatase, the most important negative regulator of Akt activity, represent relevant biomarkers to identify patients unlikely to respond to trastuzumab-based therapy (9, 10). However, in JIMT-1 cells, it has been shown that PTEN activity is retained and PTEN gene copy number ratio is unaltered (6). In the KPL-4 cell line, isolated from the malignant pleural effusion of breast cancer patients with an inflammatory skin metastasis and resistant to trastuzumab in female athymic nude mice (11), we found low levels of expression of wild-type PTEN protein (data not shown). Finally, it has been shown that resistance to trastuzumab could be related to cleavage of the full-length 185-kDa HER2 protein by matrix metalloproteases. This event produces a 110-kDa extracellular domain, which is released into cell culture media or circulates in serum in vivo (12), and a 95-kDa amino (NH2)-terminal membrane-associated fragment with increased kinase activity, defined as p95HER2 (13). Elevated serum levels of HER2 extracellular domain correlate with poor prognosis and trastuzumab resistance in patients with advanced breast cancer; in fact, HER2-targeted mAbs bind to circulating extracellular domain, competing away binding to membrane-bound HER2 (14). The p95HER2 protein may be generated by different, nonmutually exclusive mechanisms, including not only the extracellular domain cleavage but also alternative RNA processing (15) or alternative initiation of translation from different methionines within the HER2 sequence (16). However, the presence of these truncated forms of HER2 may promote resistance to trastuzumab.

Synthetic oligodeoxynucleotides containing CpG motifs similar to those found in bacterial and viral DNA act as agonists of toll-like receptor 9 and initiate a cascade of potent Th1-type innate and adaptive immune responses (17, 18). The initial impetus to develop toll-like receptor 9 agonists as anticancer drugs came from several preclinical studies showing antitumor activity in a wide variety of tumor models, in monotherapy or in combination with traditional anticancer therapies (19–24). Based on extensive structure-activity relationship studies, synthetic toll-like receptor 9 agonists containing novel DNA structures and synthetic dinucleotide motifs, referred to as immune modulatory oligonucleotides (IMOs), have been described. IMOs have been shown to induce distinct cytokine profiles in vitro and in vivo (25, 26) and showed higher metabolic stability (27) compared with conventional toll-like receptor 9 agonists. IMOs have been shown to elicit antitumor activity by inducing Th1-type innate and adaptive immune responses (28). In preclinical tumor models, IMOs enhanced antitumor activity in combination with peptide and DNA vaccines inducing Th1-type antigen-specific antibody production and cytokotoxic T-lymphocyte responses (28, 29). Previous studies with IMOs have shown potent antitumor activity in combination with chemotherapeutic agents, mAbs, and radiation (28, 30). Currently, a synthetic agonist of toll-like receptor 9, IMO-2055, is under clinical evaluation, in combination with chemotherapy and other agents in cancer patients. Although clinical development of toll-like receptor 9 agonists is very encouraging, the mechanisms by which they affect signaling proteins involved in tumor growth and angiogenesis, thus leading to tumor growth inhibition, are yet to be elucidated. Recently, we showed that an IMO acts by impairing epidermal growth factor receptor (EGFR) signaling, cooperates with anti-EGFR drugs, and boosts the antibody-dependent cell-mediated cytotoxicity (ADCC) of anti-EGFR antibodies (30). In another study, we showed that IMO interferes with tumor growth and angiogenesis also by EGFR- and ADCC-independent mechanisms, affecting endothelial cell functions (31). These new findings have opened the path to clinical studies combining toll-like receptor 9 agonists with EGFR and VEGF inhibitors in cancer patients.

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responses, as well as neoangiogenesis and mechanisms that are EGFR and ADCC dependent and independent. In the present study, we have evaluated the ability of an IMO optimized for mice to revert resistance to targeted agents other than EGFR inhibitors in tumor models different from colon cancer. Because intrinsic and acquired resistance to anti-HER2 antibody trastuzumab is an increasing concern in breast cancer treatment, we investigated whether the antitumor, antiangiogenic, and immunostimulatory activity of IMO could enhance trastuzumab effect in trastuzumab-resistant breast cancers.

Materials and Methods

Compounds. IMO, S'-TCGACRTTCT-X-TCCTRCAGTCT-5' (X and R are glycerol linker and 2'-deoxy-7-deazaguanosine, respectively), was synthesized with phosphorothioate backbone, purified, and analyzed as described previously (25, 26). The anti-HER2 mAb trastuzumab was kindly provided by Roche. The anti-VEGF mAb bevacizumab was kindly provided by Genentech.

Cell cultures. KPL-4 cell line was isolated from the malignant pleural effusion of a breast cancer patient with an inflammatory skin metastasis; in female athymic nude mice, KPL-4 are mostly resistant to trastuzumab (11). JIMT-1 cell line was established from a pleural metastasis of a 62-y-old patient with breast cancer who was clinically resistant to trastuzumab and forms trastuzumab-resistant xenografts in nude mice (6). KPL-4, JIMT-1, BT474 breast cancer cells, and human umbilical vein endothelial cells (HUVEC) were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 4% to 15% SDS-PAGE and probed with anti-human monoclonal phospho-mitogen-activated protein kinase (MAPK) and monoclonal phospho-EGFR, polyclonal EGFR, polyclonal pHER2, monoclonal HER2, and monoclonal phospho-MAPK (Santa Cruz), monoclonal pAkt, polyclonal Akt (Cell Signaling Technologies), and monoclonal actin (Sigma-Aldrich). Immunoactive proteins were visualized by enhanced chemiluminescence (Pierce), as described previously (34). Communoprecipitation analysis were done by communoprecipitation using polyclonal anti-EGFR antibody or monoclonal anti-toll-like receptor 9 antibody (Calbiochem/EMD Biosciences) and blotting with polyclonal anti-EGFR antibody or with monoclonal anti-HER2 antibody, following procedures described above. As the positive control, total proteins from KPL-4 cells were used; as the negative control, lysis buffer was mixed with anti-EGFR or anti-toll-like receptor 9 antibody.

Enzyme-linked immunosorbent assay. Anti-human VEGF polyclonal antibody (R&D Systems) diluted at 1 μg/mL in PBS (pH 7.5) was used to coat a 96-well plate, 100 μL/well, overnight at 4°C. Washings, dilutions of standards (recombinant human VEGF) and samples (conditioned media from cultured cells), biotinylation, and mixing with preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain kit) were previously described (32). The absorbance was measured at 490 nm on a microplate reader (Bio-Rad). VEGF concentrations were determined by interpolation of the standard curve using linear regression analysis.

Xenografts in nude mice. Five-week-old BALB/cAnNcHBr athymic (nu/nu) mice (Charles River Laboratories) were maintained in accordance with institutional guidelines of the University of Naples Animal Care Committee and in accordance to the Declaration of Helsinki. KPL-4 or JIMT-1 human breast cancer cells (10 6 cells per mouse) were reseeded in 200 μL of Matrigel (Collaborative Biomedical Products) and injected s.c. in mice. After 7 d, tumors were detected, and groups of 10 mice were randomized to receive the following treatments: i.p. IMO, 1 mg/kg thrice a week for 4 wk; i.p. trastuzumab, 3.75 mg/kg, twice a week for 3 wk; or the combination of these agents, on days 7 to 11, 14 to 18, and 21 to 25, continuing only IMO on days 28 to 32. Tumor volume was measured using the formula $V = \frac{1}{2} (a \times b)$, as previously reported (32). Two mice were sacrificed on day 25 to do biochemical analysis.

ADCC assay. Nonadherent fraction of human peripheral blood mononuclear cells were used as effector cells. Briefly, human peripheral blood mononuclear cells were isolated by density gradient centrifugation and resuspended in RPMI medium. Before the assay, the peripheral blood mononuclear cells were cultured 1 h on plastic dishes to remove adherent cells (monocytes), and then the peripheral blood lymphocytes were incubated for 24 h in the presence or absence of IMO (5 μmol/L). The target cells (chronic erythroid leukemia KS62 and KPL-4 or JIMT-1 breast cancer cells) were loaded with the fluorescence enhancing ligand (DELFIa BATDA reagent, PerkinElmer) and, after washing, were incubated in presence or absence of trastuzumab, 10 μg/mL. Target cells were mixed with effector cells at varying cell concentration for 2 h at 37°C and centrifuged. Supernatants were added to Europium solution, and the signal was measured as previously described (33).

Immunofluorescence and confocal microscopy analysis. Cells (5 × 10 4 per milliliter) were plated in complete medium on 12-mm diameter glass cover slips. Forty-eight hours later, cells were fixed for 20 min with 3% paraformaldehyde (Sigma) in PBS containing 0.9 mmol/L CaCl 2 and 0.5 mmol/L MgCl 2 [PBS–calcium magnesium (PBS-CM)] at room temperature and washed twice in 50 mmol/L NH 4 Cl in PBS-CM and twice in PBS-CM. Cells were permeabilized for 5 min in 0.5% Triton X-100 (Bio-Rad) in PBS-CM and incubated for 30 min in 0.5% gelatin (Sigma) in PBS-CM. Cells were then incubated for 1 h with the primary antibodies diluted in 0.5% bovine serum albumin (BSA) in PBS polyclonal rabbit anti-human EGFR (Santa Cruz) and monoclonal mouse anti-human toll-like receptor 9 (Calbiochem/EMD Biosciences). After three washes with 0.2% gelatin in PBS-CM, cells were incubated for 20 min with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:50 in 0.5% bovine serum albumin (BSA) in PBS polyclonal rabbit anti-human EGFR (Santa Cruz) and monoclonal mouse anti-human toll-like receptor 9 (Calbiochem/EMD Biosciences). After three washes with 0.2% gelatin in PBS-CM, cells were incubated for 20 min with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:50 in 0.5% bovine serum albumin in PBS. After final washes with PBS, the cover slips were mounted on a microscope slide and examined with a Zeiss 510 confocal laser scanning microscope. Samples were observed by three investigators, without knowledge of the experimental conditions.

Cell survival assay. KPL-4 or HUVEC cells were grown in 24-well plates and exposed to different doses of IMO and trastuzumab, alone or in combination. The percentage of cell survival was determined using the MTT assay, according to manufacturer’s instructions.

Vascular endothelial cell capillary tube and network formation. Five hundred microliters of diluted Matrigel was added into a 30-mm culture dish and incubated at 37°C for 30 min. After the Matrigel was solidified, HUVECs (4 × 10 3) in 1 mL of RPMI medium were added in each dish, in the presence or absence of IMO 1 μmol/L, trastuzumab 5 μg/mL, or the combination of the two agents. Cells were incubated at 37°C and photographed (+10) at 0 and 24 h. As positive control, Matrigel was mixed with VEGF 100 ng/mL (R&D Systems).

Statistical analysis. The Student’s t test and the Mantel-Cox log-rank test were used to evaluate the statistical significance of the results. All reported P values were two sided. All analyses were done with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software).
**Results**

*Trastuzumab is unable to inhibit growth and HER-related signaling pathway in KPL-4 and JIMT-1 human breast cancer cell lines.*

We have identified/selected human breast cancer cell lines, BT474, KPL-4, and JIMT-1, with different sensitivity to trastuzumab (6, 11) and characterized their sensitivity to trastuzumab in soft agar cell growth assay. As shown in Fig. 1A, BT474 cells are sensitive to trastuzumab (50% inhibitory concentration < 0.1 μg/mL), whereas KPL-4 and JIMT-1 are insensitive to trastuzumab at doses up to 50 μg/mL. Then, we have evaluated the expression level of EGFR and HER2 in these cell lines. All three types of cells show similar levels of EGFR expression, whereas HER2 levels are higher in KPL-4 and JIMT-1 than in BT474 cells (Fig. 1B). We also observed that KPL-4 cells over-express the NH2-terminal truncated HER2 receptor, p95HER2, which has been implicated in trastuzumab resistance in breast cancers (ref. 34; Fig. 1B). We have compared the effect of trastuzumab on the activation of HER signal transduction in breast cells sensitive and resistant to trastuzumab. As shown in Fig. 1C, trastuzumab reduces phosphorylation/activation of MAPK and Akt in BT474 cell line but not in KPL-4 and JIMT-1 cell lines. Similarly, trastuzumab inhibits the secretion of the main proangiogenic factor VEGF in BT474 cells but is totally ineffective in KPL-4 and JIMT-1 cells (Fig. 1D). These results suggest that trastuzumab is able to inhibit the activation of signaling proteins critical for cancer cell proliferation, survival, and angiogenesis in sensitive but not in resistant cancer cell lines.

**Combination of trastuzumab with IMO cooperatively inhibits KPL-4 and JIMT-1 breast cancer xenografts.** BALB/c nude mice xenografted with KPL-4 or JIMT-1 tumors were treated with IMO or trastuzumab, alone and in combination (Fig. 2). On day 56, 8 weeks after tumor injection, all untreated mice xenografted with KPL-4 or JIMT-1 cells reached the maximum allowed tumor size of about 2 cm³. Treatment with trastuzumab produced about 75% and 65% tumor growth inhibition in KPL-4 and JIMT-1 tumor models, respectively, on day 49, 3 weeks after treatment withdrawal (Fig. 2A and B). These data are consistent with a previous study showing that trastuzumab was able to inhibit the outgrowth of macroscopically detectable JIMT-1 xenografted tumors despite the intrinsic resistance to trastuzumab in *vitro* and that this effect was likely to be mediated through ADCC (35, 36). However, starting from the 4th week, tumors treated with trastuzumab alone resumed an exponential growth, reaching 2 cm³ size by day 98, as previously reported (35, 36). As previously shown in BT474 trastuzumab sensitive breast cancer model (37), IMO treatment inhibited tumor growth in KPL-4 and JIMT-1 xenografts that reached the 2-cm³ tumor size on days 77 and 84, respectively. The combination of IMO plus trastuzumab caused a potent and long-lasting cooperative antitumor activity, with 75% growth inhibition (tumor size of 0.5 cm³) until the end of the experiment on day 105, 11 weeks after treatment withdrawal. No treatment-related side effects were observed in either tumor model studied. Comparison of tumor sizes among different treatment groups, evaluated by the Student’s t test, was statistically significant in KPL-4 and JIMT-1 tumors (Fig. 2A and B). The median survival of mice treated with IMO or trastuzumab was 8 and 9.5 weeks, respectively, compared with 5.5 weeks in control mice. Difference among the groups was calculated by log-rank test. IMO plus trastuzumab group did not reach a median survival because 80% of the mice were still alive at the end of the experiment (Fig. 2C and D).

IMO, alone or in combination with trastuzumab, inhibits the expression of signaling proteins in KPL-4 and JIMT-1 xenografts. We studied the effect of IMO treatment on the expression of a variety of proteins playing a critical role in cancer cell...
proliferation and angiogenesis. Western blotting analysis was done on cell lysates from tumors removed at the end of the 3rd week of treatment on day 25. As shown in Fig. 3A, in KPL-4 tumors IMO induced a moderate reduction of phospho-EGFR and a more pronounced reduction of phospho-HER2. Moreover, IMO strongly inhibited the activated forms of MAPK and Akt, as well as the VEGF expression. This signaling inhibition was not observed with trastuzumab treatment; indeed, trastuzumab seemed to induce a slight activation of EGFR- and HER2-dependent signal transduction. IMO in combination with trastuzumab suppressed trastuzumab-induced signaling activation, producing a moderate inhibition compared with control. Particularly, the combination treatment is effective in reducing the expression of phospho-p95HER2, the NH2-terminal truncated HER2 receptor. No treatment affected the total amount of EGFR, HER2, MAPK, and Akt (Fig. 3A).

In JIMT-1 tumors IMO did not affect the phosphorylation/activation of EGFR, HER2, and their downstream effectors, whereas trastuzumab weakly inhibited the same signaling proteins (Fig. 3B). When the two agents were used in combination, a potient inhibition was observed on protein expression, with an almost total suppression of phosphorylation/activation of EGFR, MAPK, Akt, and VEGF expression (Fig. 3B). The combination of IMO with trastuzumab also reduced the total amount of HER2 rather than the phospho-HER2 expression levels (Fig. 3B).

IMO enhances ADCC activity of trastuzumab on KPL-4 and JIMT-1 cells. To investigate the capability of trastuzumab to activate an ADCC reaction in KPL-4 and JIMT-1 cancer cells and the influence of IMO on trastuzumab-induced ADCC when combined together, we did a cytotoxicity assay using both cell lines as targets. In the absence of antibodies, freshly isolated nonadherent human peripheral blood lymphocytes were able to kill the standard natural killer–target K562 cells but did not induce any detectable lysis of KPL-4 or JIMT-1 cells (Fig. 4A and B). Similar results were obtained when peripheral blood lymphocytes were incubated with IMO. Trastuzumab, which has been shown to induce ADCC activity against esophageal squamous cell carcinoma (38), caused a 85% (Fig. 4A) and a 35% lysis of KPL-4 and JIMT-1 cells [Fig. 4B], respectively. This difference in lysis activity could be explained on the basis of the lower HER2 expression levels and the masking of the receptor by the membrane-associated mucin MUC4 in JIMT-1 (5). Preincubation of peripheral blood lymphocytes with IMO potentiated KPL-4 and JIMT-1 cells killing induced by trastuzumab (Fig. 4A and B).

IMO interferes with EGFR signaling in KPL-4 cells by modulating a functional interaction between toll-like receptor 9 and EGFR. Because IMO treatment of KPL-4 xenografts inhibited HER-dependent signal transduction, we investigated whether this in vivo effect could be related to modulation of HER-dependent signaling on tumor cells. Therefore, we examined the effect of IMO on KPL-4 cells in vitro growth. IMO showed significant inhibition of KPL-4 cell survival (Fig. 5A). Moreover, we showed that IMO treatment of KPL-4 cells produces an inhibition of EGFR signaling also in vitro, reducing the EGFR-induced phosphorylation/activation of EGFR and MAPK (Fig. 5B).

We further did an immunofluorescence and confocal microscopy analysis for EGFR and toll-like receptor 9 on KPL-4 cells to analyze the intracellular relationship between the two receptors. As expected, EGFR was prevalently localized on cell
membranes, whereas toll-like receptor 9 seemed widely distributed in intracellular compartments and juxtamembrane regions, thus differing from the distribution described in immune cells (39). Remarkably, we observed a partial colocalization of EGFR and toll-like receptor 9 in the merge staining, suggesting the possibility of an interaction between the two receptors (Fig. 5C). To further investigate the potential functional/structural relationship between toll-like receptor 9 and HER family receptors, and the role played by IMO, we did coimmunoprecipitation experiments. First, we immunoprecipitated KPL-4 cell lysates with the anti-EGFR or the anti-toll-like receptor 9 antibody and blotted with the anti-EGFR antibody. As the positive control, total proteins from KPL-4 cells were used; as the negative control, lysis buffer was mixed with monoclonal anti-toll-like receptor 9 antibody. We showed that part of the EGFR expressed by KPL-4 cells coimmunoprecipitated with toll-like receptor 9, consistently with the partial colocalization of EGFR and toll-like receptor 9 observed in confocal microscopy analysis (Fig. 5D). We also immunoprecipitated KPL-4 cell lysates with the anti-toll-like receptor 9 anti-body and blotted with the anti-HER2 or the anti-EGFR antibody. We showed that toll-like receptor 9 coimmunoprecipitated with EGFR and HER2, but the interaction with HER2 was only slightly reduced by IMO treatment. Conversely, the interaction between toll-like receptor 9 and EGFR was strongly reduced after 1 hour of IMO treatment (Fig. 5D). These data indicate for the first time that toll-like receptor 9 functionally interacts with EGFR, that IMO is able to modulate this interaction, and that this modulation could be related to the interference with EGFR signal transduction induced in KPL-4 cells by IMO treatment (Fig. 5B).

Combination of IMO plus trastuzumab causes a direct antiangiogenic effect. To evaluate whether the strong cooperative effect of IMO plus trastuzumab combination on the growth of tumor xenografts could involve a direct antiangiogenic activity, we did a cell survival assay and a capillary tubes and network formation assay on HUVEC cells (human umbilical vein endothelial cells; Fig. 6A). These data confirmed our previous observation of IMO effects on HUVEC functions (31) and showed for the first time that trastuzumab exhibits a direct antiangiogenic effect inhibiting both HUVEC survival (20% inhibition) and capillary formation capability. As shown in Fig. 6A, the combination of IMO and trastuzumab was more effective in inhibiting HUVEC survival than the single agents. The VEGF-stimulated capillary tube and network formation was almost totally suppressed by the combined treatment (Fig. 6B). Several reports have described the expression of EGFR and HER2 receptors on HUVEC (40, 41). Therefore, we have verified whether the effect of IMO and trastuzumab...
on HUVEC functions could be related to a direct inhibition of EGFR/HER2 signaling. As shown in Fig. 6C, IMO inhibited the EGF-induced phosphorylation/activation of EGFR, HER2, Akt, and MAPK. Interestingly, trastuzumab was also able to inhibit to a similar extent the same signaling proteins, and when the two agents were used in combination, a more potent inhibition or complete suppression was observed on the expression of the proteins affected by each single agent (Fig. 6C).

Discussion

Toll-like receptor 9 agonists are a novel class of immune stimulatory agents that display a potent antitumor activity. Novel agonists of toll-like receptor 9 referred to as IMOs have been shown to induce potent antitumor activity in a number of tumor models through the activation of innate and adaptive immune responses, natural killer cell activation, cytotoxic T-lymphocyte responses, and ADCC, either alone or in combination with chemotherapeutic agents, targeted therapies, DNA vaccines, and mAbs. An IMO referred to as IMO-2055 is currently in clinical trials for cancer treatment. However, the mechanisms by which IMOs affect growth signaling and angiogenesis are yet poorly understood. Recently, we have shown that an IMO acts through EGFR and cooperates with anti-EGFR drugs, synergistically inhibiting colon cancer growth and angiogenesis. IMO is inactive against cetuximab-resistant tumors, further suggesting its dependence on EGFR signaling (30). In another study, we showed that IMO interferes with tumor growth and angiogenesis also by EGFR- and ADCC-independent mechanisms, directly affecting endothelial cell functions and synergizing with the anti-VEGF mAb bevacizumab in colon cancer xenografts sensitive and resistant to EGFR inhibitors.
In the present article, we investigated whether the anti-tumor, antiangiogenic, and immunostimulatory activity of IMO may affect sensitivity to trastuzumab in trastuzumab-resistant breast cancers. To this aim, we used HER2-overexpressing, trastuzumab-resistant KPL-4 and JIMT-1 cell lines and evaluated IMO effect on tumor growth and trastuzumab activity on KPL-4 and JIMT-1 xenografts. Interestingly, the mechanisms involved in trastuzumab resistance of KPL-4 and JIMT-1 are different. In fact, KPL-4 overexpress p95HER2, the NH2-terminal truncated HER2 fragment. It has been shown that, in a series of patients with HER2-positive advanced breast cancer treated with trastuzumab, the presence of p95HER2 is associated with clinical resistance to trastuzumab and poor clinical outcome as compared with patients with full-length receptor (38). In addition, p95HER2-expressing breast cancer cells can activate potent growth and prosurvival signals through p95HER2-HER3 heterodimers (42). Conversely to KPL-4, JIMT-1 cell line shows a decreased HER2 accessibility and a diminished trastuzumab binding due to the expression of MUC4, a membrane-associated mucin that contributes to the partial masking of HER2, which leaves only 20% of binding sites accessible in JIMT-1. Previously, it has been shown that IMO is able to increase the therapeutic efficacy of trastuzumab in BT474 breast cancer xenografts (37). In this study, we showed that the combination of IMO plus trastuzumab caused, in a similar fashion in KPL-4 and JIMT-1, a potent and long-lasting cooperative antitumor activity, with about 75% growth inhibition (tumor size of 0.5 cm³) and ~80% of the mice alive until the end of the experiment at 11 weeks after treatment withdrawal. These results show that the marked therapeutic impact of IMO plus trastuzumab is independent of the mechanism of trastuzumab resistance. Moreover, we have shown that IMO potentiates trastuzumab-mediated ADCC on KPL-4 and JIMT-1 cells, further contributing to trastuzumab activity also in resistant tumors. Because it has been shown that VEGF partially inhibits the ADCC of human monocytes mediated by trastuzumab (43), it is possible that IMO may further contribute to the

![Fig. 6. Effects of IMO and trastuzumab on HUVEC cell survival (A), tube formation (B), and signal transduction (C).](image-url)

**Fig. 6.** Effects of IMO and trastuzumab on HUVEC cell survival (A), tube formation (B), and signal transduction (C). **A,** HUVEC cells were treated with IMO at the dose of 1 μmol/L, trastuzumab 5 μg/mL, or the combination of these agents. The results are statistically significant for IMO, trastuzumab, and the combination versus control (two-sided \(P < 0.0001\)). **B,** diluted Matrigel was added into a 30-mm culture dish and incubated at 37°C for 30 min. HUVEC cells were added in presence or absence of 1 μmol/L IMO, 5 μg/mL trastuzumab, or the combination of these agents. As the positive control, the Matrigel was mixed with 100 ng/mL of VEGF. Photographs were taken at 0 and 24 h to monitor the process of vascular endothelial cell tube and network formation. **C,** Western blot analysis of protein expression in HUVEC cells cultured in serum-free medium, treated for 1 h with 1 μmol/L IMO and/or for 24 h with trastuzumab 5 μg/mL, and stimulated for 15 min with EGF 50 ng/mL before protein extraction.
ADCC effect of trastuzumab by its ability to inhibit human VEGF, as shown in this and in our previous studies. In this study, we showed a potent IMO activity on HER2 signal transduction in KPL-4 tumors in vitro and in vivo. This effect could be related to IMO capability to modulate a functional interaction between toll-like receptor 9 and EGFR in KPL-4 cells in addition to its toll-like receptor 9-mediated immune stimulation. In fact, we have shown for the first time that toll-like receptor 9 is expressed in KPL-4 cells and is widely distributed in intracellular compartments and juxtamembrane regions, differently from the distribution described in immune cells. In human B cells, toll-like receptor 9 is retained in the endoplasmic reticulum before exposure to toll-like receptor 9 agonists, and then, it is translocated to endosomal compartments that contain the agonists. However, toll-like receptor 9 may reside in a different cellular compartment/location depending on cell type, as previously described also for toll-like receptor 4. It has also shown that another endosomal toll-like receptor, toll-like receptor 3, also may be located in different cellular compartment (44). Recent studies have shown that double-stranded RNAs that act as ligands of toll-like receptor 3 interact with cell surface toll-like receptor 3 and induce antiangiogenic effects (44, 45). EGFR acts as a negative regulator for another toll-like receptor, toll-like receptor 2, through a Src- and MAPK-dependent mechanism, attenuating host immune response (46), and has been involved also in the induction of toll-like receptor 4 and 2 expression (47). Finally, multiple toll-like receptor ligands have shown to induce airway epithelial cell production of IL-8 and VEGF through a signaling cascade involving EGFR (48). In this study, we showed partial colocalization and a biochemical functional interaction between toll-like receptor 9 and EGFR at membrane level and provide evidence that this interaction is modulated by IMO, eventually producing an inhibition of EGFR downstream signaling. Together, these data strongly suggest that disruption of EGFR–toll-like receptor 9 interaction may contribute to inhibit mitogenic signaling for breast tumor cells. Because several studies have suggested the existence of a potential toll-like receptor 9 coreceptor at the plasma membrane, able to interact with CpG before the activation of endosomal toll-like receptor 9 (49), further investigations dissecting all the relationships between EGFR and toll-like receptor 9 could elucidate the role played by EGFR in this process.

Several reports have shown that, in multiple carcinomas, HER receptors are expressed and activated not only in tumor cells but also in tumor-associated stroma, particularly in tumor vasculature (40, 41). The functional role of HER receptors expressed by stromal cells has been shown in a recent study indicating the activation of EGFR on tumor-associated endothelial cells as a major determinant for the susceptibility of human cancers to EGFR–tyrosine kinase inhibitors (50). Consistently with this evidence, we have shown that trastuzumab exhibits a direct antiangiogenic effect inhibiting endothelial cell survival and capillary tubes formation and that the cooperative antiangiogenic effect obtained with the combination IMO plus trastuzumab is likely mediated by a direct inhibition of EGFR/HER2 signaling in endothelial cells.

In conclusion, in this study, we show that IMO strongly cooperates with trastuzumab producing a potent and long-lasting growth inhibition in trastuzumab-resistant breast cancer xenografts. The cooperative effect is due to antitumor and antiangiogenic activity of IMO, particularly when used in combination with trastuzumab, and to the enhancement of trastuzumab-induced ADCC. In addition, we provide here the first evidence that toll-like receptor 9 and EGFR could interact at membrane level and uncouple following IMO treatment. This event may explain the potent inhibitory effect of IMO on HER-mediated signaling. Here, we showed a novel mechanism of action of IMO in addition to its toll-like receptor 9 mediated immune stimulation. Because IMO is currently in clinical development, our results support the possible use of IMO plus trastuzumab as an effective therapeutic strategy in trastuzumab-resistant breast cancer patients.

Disclosure of Potential Conflicts of Interest

S. Agrawal and E. Kandimalla are employed by and G. Tortora is a consultant for Idera Pharmaceuticals.

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